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Claim 1 is unobvious over Song et al. in view of Hedley et al. and Fattal et al.

As previously noted, Song et al. proposes several gene delivery techniques.

According to Song et al., direct injection of recombinant retroviruses is preferred. See, e.g., Song et al., page 27, lines 25-27. Moreover, among the non-viral techniques taught by Song et al., none appears to use a transfection agent comprising a polynucleotide and microparticles as claimed.

Recognizing this, the Office contends that Hedley et al. supplements Song et al. by teaching the use of microspheres comprising biodegradable polymers and DNA plasmids to introduce and express antigens encoded by the plasmids "in antigen presenting cells such as macrophages and dendritic cells." The Office further contends that Hedley et al. provides motivation to introduce plasmid DNA encoding an antigen to "dendritic cells and macrophages" by teaching that DNA combined with biodegradable microparticles is efficiently phagocytosed by APCs.

However, such motivation exists only with the benefit of the hindsight afforded by the present application.

First of all, it is respectfully submitted that one of ordinary skill in the art would not have been motivated to turn from the techniques of Song to microparticle techniques such as those taught by Hedley et al., particularly in view of Song's preference for recombinant retroviral techniques, and in view of the notorious resistance of dendritic cells to transfection using *ex vivo* nonviral techniques (discussed further below).

Moreover, dendritic cells are mentioned only a single time in all of Hedley et al., where it is observed in passing that, were the microparticles of Hedley et al. to be delivered intradermally, the microparticles would be *introduced* to dendritic cells that are inherently present in the skin--hardly motivation for transfecting dendritic cells *ex vivo* as claimed in claim 1. See, Hedley et al., col. 8, lines 20-34:

The microparticles can be delivered directly into the bloodstream (i.e., by intravenous or intraarterial injection or infusion) if uptake by the phagocytic cells of the reticuloendothelial system (RES) is desired. Alternatively, one can target, via subcutaneous injection, take-up by the phagocytic cells of the draining lymph nodes. The microparticles can also be introduced intradermally (i.e., to the APCs of the skin, such as *dendritic cells* and Langerhans cells). Another useful route of

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delivery (particularly for DNAs encoding tolerance-inducing polypeptides) is via the gastrointestinal tract, e.g., orally. Finally, the microparticles can be introduced into the lung (e.g., by inhalation of powdered microparticles or of a nebulized or aerosolized solution containing the microparticles), where the particles are picked up by the alveolar macrophages.

Elsewhere, including the procedure set forth in Example 2, Hedley et al. is concerned with the phagocytosis and expression of DNA by *macrophages*. In this regard, the Office appears to be under the mistaken impression that successful *in vitro* phagocytosis and expression within macrophages would provide a reasonable expectation of success within dendritic cells. This, however, is not the case. As previously noted, dendritic cells are notoriously resistant to transfection using *ex vivo* nonviral techniques. See, e.g., the present specification at page 6, lines 6-10. See also the Abstract [copy attached] of Denis-Mize K.S., et al., *Gene Ther* 2000 Dec;7(24):2105-12, which notes that "DC [dendritic cells] are not readily transfected *in vitro* by traditional nonviral techniques." See further Lundquist et al. [copy attached] *Journal of Immunotherapy*, 25(6):445-454, 2002, which confirms that dendritic cells are difficult to transfect using non-viral methods. Note that attempts with cationic lipids and gold beads, even where 1 μ m in size, were not successful.

In addition, from a therapeutic standpoint, Hedley et al. as a whole is clearly directed to *in vivo* treatment techniques. Example 2 of Hedley et al. does describe an *in vitro* procedure, but this is commonly the case with patents that are directed to *in vivo* therapies and therefore would not have necessarily motivated one of ordinary skill in the art to pursue an *ex vivo* therapeutic strategy.

Furthermore, and in contrast to claim 1, Hedley et al. does not teach or suggest a transfection agent comprising polynucleotide *adsorbed* on surfaces of microparticles, which is formed by a process that comprises exposing previously formed microparticles to the polynucleotide. Rather Hedley *encapsulates* the polynucleotide within the polymeric matrix of the microparticle. See, e.g., Hedley Abstract, col. 9, lines 2-4 ("microparticles can be prepared which carry ... DNA ... *within* each microparticle"), col. 13, lines 64-66 ("the protein or peptide encoded by the nucleic acid contained *within* the microparticle"), Table 5 ("[p]hagocytosis of *encapsulated* DNA leads to expression of

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a luciferase reporter gene construct") and Table 6 ("[e]xpression of *encapsulated* luciferase DNA in murine muscles"). (Emphasis added.)

Fattal et al. does not make up for the above-noted deficiencies in the combination of Song with Hedley et al. The Office Action maintains that one of ordinary skill in the art upon reading Fattal et al. would include a cationic detergent such as CTAB in the microparticles of Hedley et al. in order to increase the amount of polynucleotide associated with the polymer particles and increase the uptake of the nucleic acid by phagocytosis. However, as above, this is true only with resort to the hindsight afforded by the present application.

First, Fattal et al. and Hedley et al. are directed to entirely different ways of introducing polynucleotide into cells. While Fattal et al. associates antisense oligonucleotide with the surfaces of previously formed polymer particles, Hedley et al., on the other hand, *encapsulates* polynucleotide within the microparticles. Hence, contrary to the Office Action, one of ordinary skill in the art would not be motivated to modify the microparticles of Hedley et al. by the addition of the cationic detergent from Fattal et al. to increase the association of the polynucleotide with the microparticles, because there is no teaching or suggestion that Hedley et al., with its contrary strategy of encapsulating polynucleotide, would benefit from the use of a cationic detergent.

In fact, one of ordinary skill in the art would have found various reasons to *avoid* the use of a cationic detergent. For example, detergents are typically added to stabilize emulsions that are commonly used to prepare microparticles and to impart desirable physical properties to the finished microparticle powder preparation, for example, the ability to flow freely. Nonionic detergents, in particular, polyvinyl alcohol are commonly used for this purpose (see, e.g., Example 1 of Hedley et al.). Charged detergents, on the other hand, are less desirable, because they impart undesirable properties such as stickiness to the resulting microparticles. For this reason, one of ordinary skill in the art would have been motivated to avoid the use of cationic detergents such as CTAB.

The motivation to avoid cationic detergents would have been reinforced by the fact that nonionic detergents, such as polyvinyl alcohol, are generally known to have reduced toxicity as compared to cationic detergents, such as CTAB.

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Furthermore, Fattal et al., which is directed to the delivery of antisense oligonucleotides, does not teach or suggest the delivery of a polynucleotide that encodes and expresses an antigen associated with a virus, a bacterium, a parasite, a fungus or a tumor as presently claimed in claim 1.

Indeed, it is not at all obvious that a preparation that delivers adsorbed antisense oligonucleotides for the purpose of binding to and inactivating messenger RNA (e.g., in the treatment of liver diseases--see Fattal et al. Abstract) would also be effective at delivering adsorbed DNA for the purpose of transcription and translation. In this regard, it is noted that antisense oligonucleotides are generally prepared with backbones other than deoxyribonucleic acid in order to prevent degradation by endonucleases within the target cell. Many practitioners, for instance, use phosphorothioate linkages rather than the phosphodiester linkages found in native DNA. However to establish transfection it is essential that the unmodified deoxyribonucleic acid backbone be used.

In addition, as with Hedley et al. above, the asserted success of Fattal et al. in connection with the delivery of antisense oligonucleotides to U937 cells (commonly referred to in the art as "monocyte-like" or "macrophage-like" cells) would not lead to a reasonable expectation that dendritic cells can be successfully transfected with DNA, because, as noted above, dendritic cells are notoriously difficult to transfection using non-viral methods.

Finally, in claim 1 as presently presented, the transfection agent is formed by a process that comprises: (a) providing microparticles comprising a biodegradable polymer and a cationic detergent, and (b) exposing the microparticles to a polynucleotide. Neither Song nor Hedley et al. nor Fattal et al. teaches or suggests such a process. (Note that Fattal et al. teaches admixing oligonucleotides with detergent *prior to* exposure to nanoparticles.)

For at least the above reasons, it is respectfully submitted that a *prima facie* case of obviousness has not been established with respect to the presently pending claim 1. Claims 2-16, 18-23, 29-31, 33-44, 46, 50, 52 and 53 depend from claim 1 and are therefore patentable for at least the same reasons as is claim 1. Claims 17, 24-28, 32, 45, 47-49 and 51 have been deleted.

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Reconsideration and withdrawal of the outstanding rejection of claims 1-23 and 29-53 under 35 U.S.C. §103(a) are therefore respectfully requested.

CONCLUSION

All pending claims are in condition for allowance, notification of which is earnestly solicited. The Examiner is invited to telephone the Applicant's attorney at (703) 433-0510 to resolve any outstanding issues in this case.

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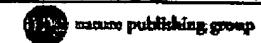
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 1: Gene Ther. 2000 Dec;7(24):2105-12.[Related Articles](#), [Links](#)**Plasmid DNA adsorbed onto cationic microparticles mediates target gene expression and antigen presentation by dendritic cells.****Denis-Mize KS, Dupuis M, MacKichan ML, Singh M, Doe B, O'Hagan D, Ulmer JB, Donnelly JJ, McDonald DM, Ott G.**

Department of Anatomy, and Cardiovascular Research Institute, University of California San Francisco, San Francisco, CA, USA.

Dendritic cells (DC) play a key role in antigen presentation and activation of specific immunity. Much current research focuses on harnessing the potency of DC for vaccines, gene therapy, and cancer immunotherapy applications. However, DC are not readily transfected *in vitro* by traditional nonviral techniques. A novel DNA vaccine formulation was used to determine if DC are transfected *in vitro*. The formulation consists of plasmid DNA adsorbed on to cationic microparticles composed of the biodegradable polymer polylactide-co-glycolide (PLG) and the cationic surfactant, cetyltrimethylammonium bromide (CTAB). Using preparations of fluorescent-labeled plasmid DNA formulated on PLG-CTAB microparticles to study internalization by macrophages and dendritic cells *in vitro* and *in vivo*, we found that most, but not all, of the fluorescence was concentrated in endosomal compartments. Furthermore, uptake of plasmid DNA encoding HIV p55 gag adsorbed to PLG-CTAB microparticles by murine bone marrow-derived dendritic cells resulted in target gene expression, as detected by RT-PCR. The antigen was subsequently processed and presented, resulting in stimulation of an H-2kd-restricted, gag-specific T cell hybridoma. Activation of the hybridoma, detected by IL-2 production, was dose-dependent in the range of 0.1-20 microg DNA (10-2000 microg PLG) and was sustained up to 5 days after transfection. Thus, adsorption of plasmid DNA on PLG-CTAB microparticles provides a potentially useful nonviral approach for *in vitro* transfection of dendritic cells. *Gene Therapy* (2000) 7, 2105-2112.

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Nonviral and Viral Gene Transfer Into Different Subsets of Human Dendritic Cells Yield Comparable Efficiency of Transfection

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Summary: Among the many promising cancer immunotherapeutic strategies, dendritic cells (DC) have become of particular interest. This study aims to optimize a clinical grade protocol for culture and transfection of human DC. Monocytes and CD34⁺ hematopoietic stem cells (HSC) from same donor were differentiated under serum-free conditions and analyzed for their susceptibility to several recently described nonviral transfection methods as compared with established virally mediated gene transfer. Nonviral gene transfer methods studied were square-wave electroporation, lipofection, and particle-mediated transfer of plasmid DNA or in vitro transcribed mRNA. We conclude that DNA is not suitable for transduction of DC using nonviral methods. In contrast, mRNA and square-wave electroporation reproducibly yields 60% and 50% transfected monocyte- and CD34⁺-derived DC, respectively, measured at protein level, without affecting the cell viability. Thus, the transfection efficiency of this method is comparable with the 40-90% transgene expression obtained using retroviral (RV) or adenoviral (AdV) vectors in CD34⁺- and monocyte-derived DC, respectively. In monocyte-derived DC, however, the amount of protein expressed per-cell basis was higher after AdV (MOI = 1000) compared with mRNA electroporation-mediated transfer. This is the first study directly demonstrating side-by-side that mRNA electroporation into DC of different origin indeed results in a comparable number of transduced cells as when using virus-mediated gene transfer. **Key Words:** Dendritic cells—Electroporation—Gene transfer—Transfection efficiency.

Dendritic cells (DC) are the most potent antigen-presenting cells (APC) and have the unique capability of activating CD4⁺ and CD8⁺ naive T lymphocytes, leading to the induction of primary immune response (1-4). APC, such as, B cells and macrophages, are typically incapable of inducing primary responses. DC derive their stimulatory potency from high-constitutive and upregu-

lated expression of major histocompatibility complex (MHC) class I, MHC class II, and accessory molecules, such as, CD40, CD54, CD80, CD86, and T-cell activating cytokines, all directly or indirectly involved in the antigen presentation and by providing the essential secondary signals for the initiation of the primary immune response (5). These unique characteristics combined with developments of in vitro methods for generation of large numbers of DC from CD34⁺ hematopoietic stem cells or peripheral blood mononuclear cells (PBMC) make DC a suitable candidate for immunotherapy against infectious and malignant diseases (6-9).

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Results obtained in animal studies and clinical trials confirm that DC are capable of inducing an antigen-specific cytotoxic T lymphocyte (CTL) response, resulting in strong immunity to viruses and tumors and, in some instances, regression of established tumors (10–14). These studies include injections of tumor antigen protein or peptides either alone or in combination with various adjuvants, genetically modified tumor cells, plasmid DNA encoding tumor antigens, and DC manipulated to present specific tumor antigens (12,15–21). It has recently been shown that DC can be efficiently transfected using square-wave electroporation and *in vitro* synthesized mRNA and that such DC are capable of inducing CTL responses *in vitro* (22). Among these different immunotherapeutic strategies, we focus on approaches using DC genetically engineered to express an entire antigen.

Expression of genes in DC provides important advantages over peptides: (i) their application does not require prior knowledge of patient HLA haplotype or specific T-cell epitopes; (ii) the expression of the entire antigen within the APC that allows for the concurrent processing and presentation of multiple but undefined tumor antigens of clinical importance; and (iii) antigen expression from a transgene can extend the duration of antigen presentation. Furthermore, the transfer of the antigen from tumor cells to DC is implicated as one of the limiting factors of tumor-specific CTL induction *in vivo*, and direct loading of DC with the relevant antigen is an effective method to bypass this process. Variability in antigen loading, route of vaccination or origin of DC makes it difficult to compare immunologic and clinical results. Considering that only a fraction of the studied patients have tumor regression suggests that further improvement is required.

In the current study, we compared the transfection efficacy using viral and nonviral delivery methods of monocyte- and CD34⁺-derived DC. These two different types of DC have been used in immunization trials and are characterized by their potent capacity to stimulate alloreactive T cells, but it is not yet clear which type of DC is more suitable for efficient gene transfer. Several recent studies have described a variety of nonviral transfer methods among which especially mRNA and electroporation resulted in superior gene transfer. Nevertheless, the general belief persists that viral vectors are the method of choice to transduce human DC. For the first time, our results demonstrate that mRNA and square-wave electroporation yields a comparable level of transfection efficiency as when using virus-mediated transfer without affecting cell viability.

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MATERIALS AND METHODS

Patients and Healthy Donors

All patient and volunteer blood samples were acquired after informed consent according to the guidelines of the Institutional Review board (IRB) and the Helsinki protocol.

Preparation of Dendritic Cells

Monocytes and CD34⁺ progenitor cells were isolated from the same patients by leukapheresis. CD34⁺ cells were isolated by positive selection using the Isolex bead separation technique (generous gift from Nexell Therapeutics Inc., Irvine, CA, U.S.A.) from patients treated with 5–10 µg/kg G-CSF (Neupogen; Amgen, Stockholm, Sweden), administered subcutaneously for 5 days. The purity of CD34⁺ cells ranged from 75% to 95%. Selected cells were frozen in liquid nitrogen in 90% autologous or blood group compatible plasma with 10% DMSO (10×10^6 cells/mL). Purified cells were cultured in X-vivo15 (Biowhittaker, Rockland, ME, U.S.A.) in presence of 50 ng/mL GM-CSF (Leucomax; Schering-Plough, Kenilworth, NJ, U.S.A.) and 10 ng/mL tumor necrosis factor (TNF)- α (Chiron Corporation, Emeryville, CA, U.S.A.). Where indicated, LPS (Sigma, St. Louis, MO, U.S.A.) was added to push cells to terminal differentiation on day 7 at a concentration of 100 ng/mL. Cells were vigorously washed on day 8 before use in functional studies. Monocytes were enriched by negative depletion using immunomagnetic selection (monocyte isolation kit; Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions.

Alternatively, leukapheresed blood from patients or PBMC from healthy donors were isolated by plastic adherence. Monocytes were cultured in X-vivo15 (Biowhittaker) medium supplemented with 50 ng/mL GM-CSF and 40 ng/mL IL-4 (Schering-Plough) for 8 days. Medium was changed every 2–3 days. To isolate mature DC, 50 ng/mL TNF- α was added 24 hours before harvesting the cells.

Antibodies and Flow Cytometry

The generated DC were characterized by flow cytometry using a FACScan or FACSCalibur cytometer (Becton Dickinson, San José, CA, U.S.A.) on day 8. The following panel of mAbs was used: Fluorescein (FITC)-conjugated anti-CD3, anti-HLA-DR, anti-CD80, and anti-CD1a; Phycoerythrin (PE)-conjugated anti-CD86, anti-HLA-ABC, anti-CD56, and anti-CD19 (PharMin-

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gen, Hamburg, Germany); PC5-conjugated anti-CD14 and PE-conjugated anti-CD83 (Coulter Immunotech, Marseille, France). All antibodies were used with appropriate isotype controls.

Expression of β -galactosidase in transfectants was characterized by flow cytometry using a FluoReporter lacZ Flow Cytometry Kit (Molecular Probes, Leiden, The Netherlands) according to the manufacturer's instructions. Expression of the GFP transgene was characterized by flow cytometry using the instrument set for fluorescein detection. Alternatively, GFP expression was visualized with a Zeiss fluorescent microscope using the fluorescein filter set. Viability of the cells was assessed by trypan blue exclusion or by propidium iodide (PI) staining (Molecular Probes).

Mixed Leukocyte Reaction

Following the manufacturer's instructions, a Pan T Cell Isolation Kit (Miltenyi Biotech) separated the allogeneic T cells. Stimulator cells were irradiated (25 Gy) and cocultured with 100,000 T cells. After 5 days, 1 μ Ci of H^3 -thymidine was added to each well and incubated for 12–16 hours. Cells were harvested and measured in scintillation counter (1450 MicroBeta; Wallac, Turku, Finland). Stimulation index (SI) is calculated by CPM values for the net stimulation divided by CPM values for responder cells alone.

Plasmids

The commercially available plasmids—pcDNA3.1/Hygro/LacZ, designated pcDNA-lacZ and pcDNA3.1/Hygro, designated pcDNA3 (Invitrogen, Leek, The Netherlands)—were used for transfection by nonviral methods. Furthermore, to produce mRNA with polyA tail, plasmid pSP6-IRES-EGFP was constructed by inserting EGFP gene from pIRES2-EGFP plasmid (Clontech, Palo Alto, CA, U.S.A.) into MCS of the pSP64 (polyA) plasmid (Promega, Madison, WI, U.S.A.). mRNA was prepared by in vitro transcription using RiboMax kit (Promega) with 5'-methylated cap-structure (New England Biolabs, Beverly, MA, U.S.A.), purified using RNeasy purification kit (QIAGEN, Valencia, CA, U.S.A.) and concentration determined by absorption at 260 nm.

Nonviral Gene Transfer Techniques

Electroporation

Electroporation was performed with two different square-wave pulse generators, Electro Cell Manipulator,

model ECM830 (Genetronics, San Diego, CA, U.S.A.) and PA-4000 pulse generator (Cyto Pulse Sciences, Columbia, MD, U.S.A.). The electroporation was performed at 20°C with 1×10^6 cells resuspended in 200 μ L fresh medium and electroporated in electroporation cuvettes with electrode-gaps of 0.1–0.2 mm at 20°C. DNA and mRNA amount ranged between 10–50 μ g. The electroporation parameter for both methods was 20–800 V and 0.25–100 ms pulse length. Viability of cells after electroporation was always greater than 80% as analyzed by trypan-blue staining or PI staining. Transfected cells were analyzed by histochemical staining or flow cytometry 12–48 hours after transfection. All non-viral transfection methods resulted in high percentage (> 50%) into cos-7 cells or 293 cells, which served as positive control.

Lipofection

For lipofection, several different commercially available cationic liposomes, including DOTAP and DOSPER (Boehringer Mannheim, Mannheim, Germany), were used according to the manufacturer's protocols. Transfections were performed in 6-well plates using 1×10^6 cells in 1 mL serum-free OptiMEM (Life Technologies). Different amounts of liposomes (3–24 μ g) and plasmid DNA (1–5 μ g) were used in an attempt to determine optimal conditions. Liposomes were diluted in 100 μ L OptiMEM mixed with plasmid DNA, also diluted in 100 μ L OptiMEM. After incubation for 15 minutes at 20°C, the lipid-DNA mixture was added to the cells, mixed gently, and incubated for 6 hours. Thereafter, the medium was replaced with fresh culture medium. Transfected cells were analyzed 12–36 hours after transfection.

Particle-Mediated Gene Transfer

0.5 mg of gold beads (1.0 or 1.6 μ m in diameter) was coated with 0.8 μ g/shot plasmid DNA by treatment with $CaCl_2$ and spermidine as described by others (23,24). After washing of the DNA-coated beads with ethanol, the beads were spread onto macrocarrier discs. On the day of transfection, 1×10^6 cells were spread in 35-mm dishes. Shortly before the bombardment, the medium was removed, and DNA-coated beads were delivered into cells using a Biostatic PDS-1000/He System (Bio-Rad, Hercules, CA, U.S.A.) Helium pulse ranged between 650–1100 psi. Twenty-four hours after transfection, X-Gal staining detected the transgene. Transfection of the "empty" plasmid without the lacZ gene was used as control. Trypan-blue staining assessed viability.

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Viruses and Viral Transfer

The adenovirus AdV/GFP used in this study is an E1/E3-replication-deficient vector containing the Green Fluorescence Protein (GFP) gene under the control of the strong cytomegalovirus promoter. Viral seed stocks were obtained by propagation in 293 cells. Titer of each viral stock ranged from 5×10^9 to 5×10^{10} pfu/mL, as determined by plaque assay on 293 cells. Virus was added to the DC at varying MOIs, ranging from 10 to 1000, and incubated for 48 hours. Transduced cells were evaluated by flow cytometry and fluorescence microscopy.

A retroviral construct containing the *E. coli* lacZ gene under the LTR promoter control was packaged in three packaging cell lines of different origins: the amphotropic dog cell line (DA), the amphotropic human cell line

(HA), and the xenotropic human cell line (2X) (25). These constructs were designated as DA/βgal, HA/βgal, and 2X/βgal, respectively. Retroviral transduction was performed using centrifugation method (TC Fong, personal communications). Briefly, 2.5×10^5 DC/mL were resuspended in X-vivo15 (Biowhittaker) fresh medium and seeded in 24-well plates. Virus-containing supernatant were added to each well at a MOI of 5–10 in presence of 10 µg/mL dextran sulfate. Cells were incubated for 8–16 hours at 37°C followed by centrifugation at 1000 rpm for 1 hour at 32°C. Incubation was continued at 32°C for 20 hours. Medium was then aspirated, and the same procedure was repeated to achieve higher transduction. Transduction efficiency was evaluated as described below, using the LacZ flow cytometry kit (Molecular Probes). Transduction of K562 cells was included

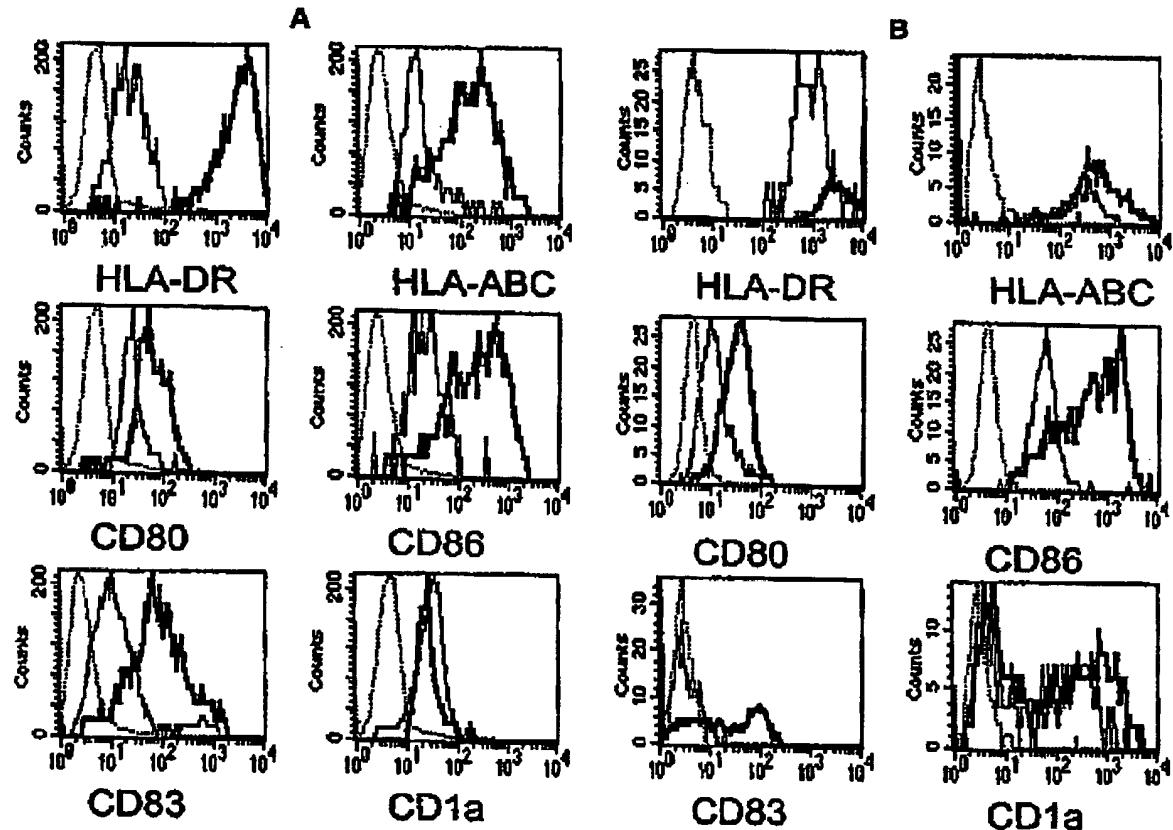


FIG. 1. Phenotype of monocyte- and CD34⁺-derived dendritic cells (DC). Immunomagnetically purified (A) CD34⁺ and (B) monocytes were differentiated into DC. Cells were harvested on day 8 and analyzed for common DC markers. Expression of all markers except CD1a was upregulated upon tumor necrosis factor- α (for monocyte-derived DC) or LPS (for CD34⁺-derived DC) treatment. Dotted lines represent cells stained with isotype of non-DC population (5%), mainly consisting of CD3⁺ cells, was not included in analysis. Results depict one of four independent experiments.

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as positive control in all experiments and ranged between 95–100%.

Statistical Analysis

Student *t* test was performed. A value of $p \leq 0.05$ was considered significant. The Ethics committee of the Karolinska Hospital approved the study.

RESULTS

CD34⁺ cells and monocytes were isolated from four different patients and further purified by immunomagnetic selection or, in some cases, by plastic adherence (for monocytes). When using immunomagnetic selection method, the purity of monocytes and CD34⁺ cells was always greater than 95% and 75%, respectively. The non-DC population mainly consisted of CD3⁺ cells. Monocyte- and CD34⁺-derived DC were treated with TNF- α and LPS for final maturation, respectively. Both subsets of DC, harvested on day 8 of differentiation, displayed a characteristic DC morphology as well as high expression of typical DC markers (Fig. 1). Upon maturation with TNF- α or LPS, all surface markers, apart from CD1a, were upregulated on both types of DC.

Gene Transfer Using Nonviral Methods

Three established nonviral gene transfer methods to introduce plasmid DNA or mRNA into DC were evaluated: square-wave electroporation, lipofection, and particle-mediated transfer.

The electroporation system was a square-wave electroporator, which, in our hands, is more efficient in transfecting cells and less damaging to the cells compared with exponential wave electroporation (22). mRNA transduction with a construct containing polyA tail resulted in high percentage of transduced cells of monocyte- and CD34⁺-derived DC, 60% and 40%, respectively, at 800–1000 V and 0.25 ms (Figs. 2A and B). GFP expression remained stable for 48 hours after electroporation. Viability was always greater than 80% for both DC subsets. Furthermore, these DC maintained their allostimulatory capacity (data not shown). Using the same parameters as for DC, undifferentiated monocytes (day 1) could not be transfected (data not shown). In contrast, the above-mentioned conditions resulted in 50% transduced 293 cells (data not shown). Electroporation with DNA resulted in no detectable transgene expression. Furthermore, the viability dropped dramatically after DNA electroporation.

For liposome-mediated transfer, three different liposomes were tested: DOSPER, DOTAP, and lipofectin.

Using mRNA and lipofectin, we were able to detect 1–10% positive monocyte-derived DC (Fig. 3C). In contrast, using the above-mentioned conditions resulted in 60% transduced 293 cells (data not shown). Despite the wide range of amount of liposomes (3–24 μ g) and plasmid DNA (1–5 μ g), no transduction of DC using DNA was detected (data not shown).

The transfection efficiency of the electroporation and lipofection method was compared with particle-mediated transfer using the Biolistic system, which is considered a powerful method of delivering DNA into cells. DC of

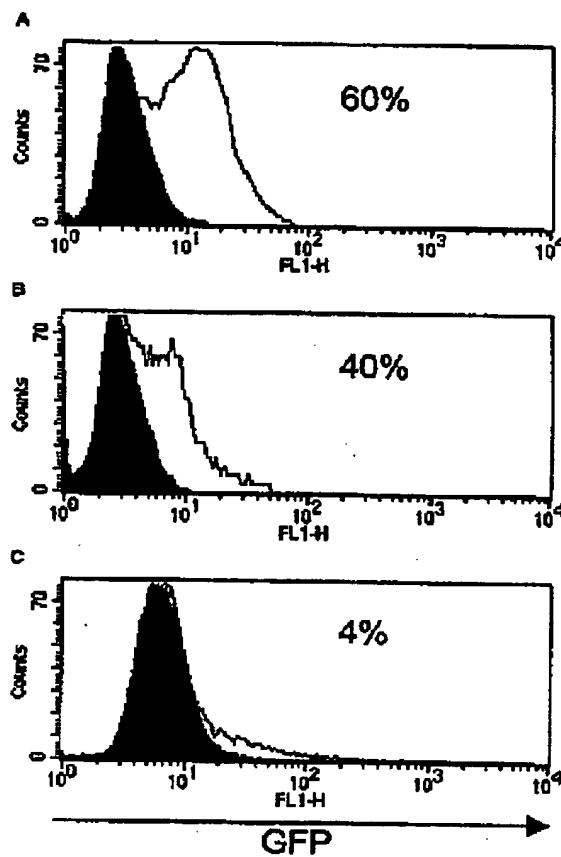


FIG. 2. Nonviral transfection of monocyte- (A) and CD34⁺-derived (B) dendritic cells (DC), CD34⁺- and monocyte-derived DC were transfected on day 7 with 50 μ g of mRNA (pSP6-IRES-EGFP) using electroporation and analyzed for GFP expression 24 hours after transfection by flow cytometry. (C) Lipofection of monocyte-derived DC. Y-axis corresponds to mean fluorescence intensity for GFP. Cells analyzed in histogram were positive for CD86 and major histocompatibility complex class I. A small fraction of non-DC population (5%) was not included in analysis. Results depict one of three independent experiments.

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both origins were bombarded on different days during differentiation (days 1–7). This yielded between 1–2% transfected cells on days 5 and 7 using pressures of 650 and 900 psi (data not shown). Only cells cultured in TNF- α containing medium were susceptible to transfection. Bombardment of cells from earlier time points did not result in any positive transfectants independent of the presence of TNF- α . Increasing pressures to 1100 psi did not result in measurable expression and exceeding 1800 psi resulted in decreased cell viability (data not shown).

Gene Transfer Using Viral Methods

Next, we examined the transduction efficacy using two of the most commonly used viral vectors: retroviral and adenoviral based vectors. CD34 $^{+}$ -derived DC were susceptible to RV transduction by constructs DA/ β gal, HA/ β gal, and 2X/ β gal, 58 \pm 4, 27 \pm 5, and 23 \pm 10%, respectively (MOI = 10). Although the CD34 $^{+}$ precursor cells undergo substantial proliferation and persistent maturation during culture, the transduction efficiency remained unchanged between days 1 through 6 (Fig. 3A). Cell proliferation and viability was unaffected by RV transduction (data not shown). Monocyte-derived DC were not susceptible to RV transduction, whereas K562 cell line, which served as positive control, was transduced to 95% under same conditions.

Monocyte-derived DC were exposed to AdV at MOI ranging from 10–1000, which resulted in 7 \pm 3% to 65 \pm 5% transduced cells (Fig. 3B). Additional co-culture with AdV for a total of 72 hours resulted in upregulation of GFP positive DC to 90 \pm 13% without affecting the viability (> 95%). In contrast, transduction of CD34 $^{+}$ -derived DC by AdV at MOI of 1000 yielded transfection efficacy of 22 \pm 6% 48 hours after transfection (Fig. 3C).

While the percentage of transfected monocyte-derived DC, using AdV or mRNA electroporation, was comparable, the level of transgene expression was different. Side-by-side analysis revealed that AdV-transduced monocyte-derived DC exhibited significantly higher

GFP expression than their mRNA electroporated counterparts (MFI 309 \pm 120 and 83 \pm 31, respectively) (Fig. 4). In the case of CD34 $^{+}$ -derived DC, which expressed lower levels of GFP after AdV transfer (MFI 154 \pm 54),

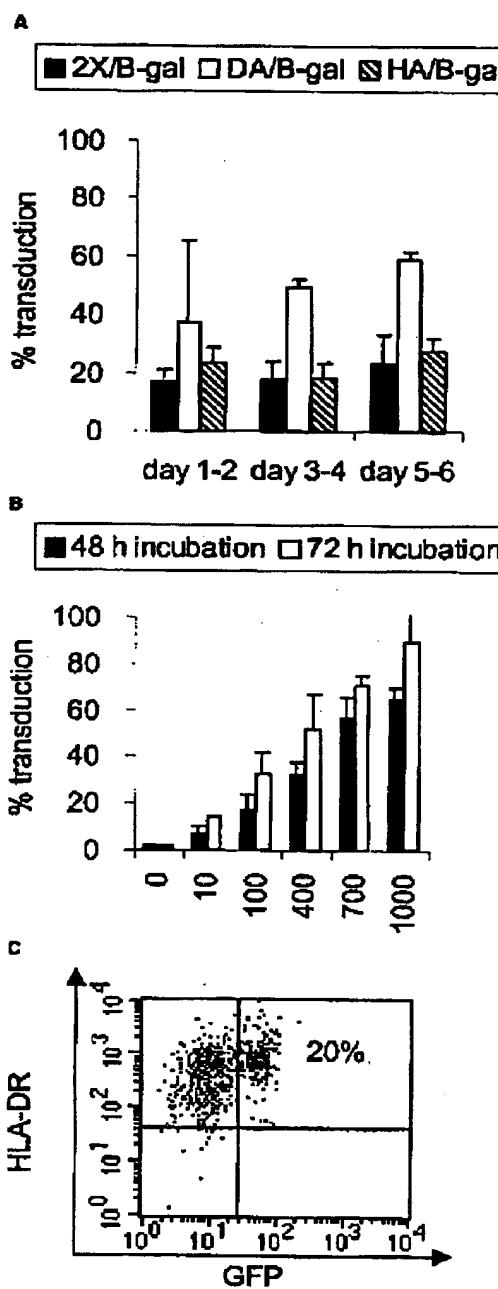


FIG. 3. Viral transduction of monocyte- and CD34 $^{+}$ -derived dendritic cells (DC). (A) Purified CD34 $^{+}$ cells were transduced with three different RV constructs (2X, DA, and HA) at a MOI of 10 on different days during differentiation (days 1–2, 3–4, and 5–6) using the centrifugation methods with two rounds of transduction. Expression of β -galactosidase was measured 24 hours after the second round of transduction by flow cytometry. Cells positive for β -galactosidase were also positive for CD86 and major histocompatibility complex class I. Standard deviation is calculated from four independent experiments. (B) Adenoviral transduction of monocyte-derived DC at increasing MOI and (C) adenoviral transduction of CD34 $^{+}$ -derived DC at a MOI of 1000. Expression of GFP expression was analyzed 24–48 hours after transduction by flow cytometry. Results depict one of four independent experiments.

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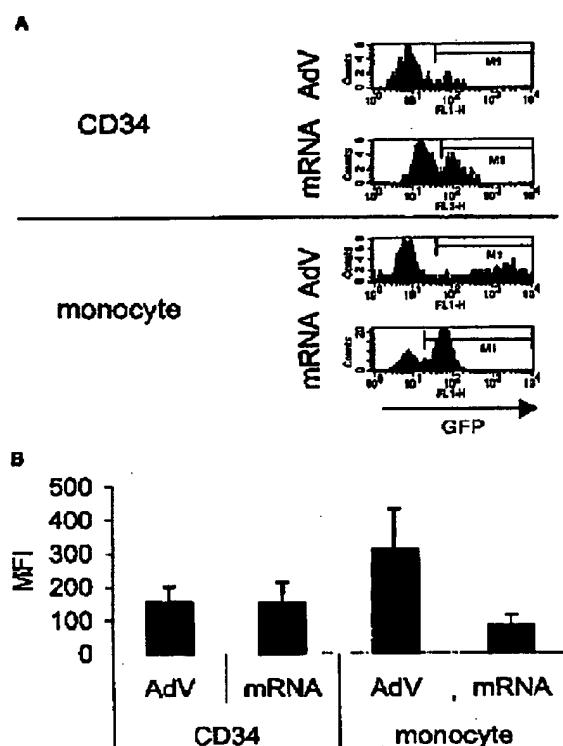


FIG. 4. Comparative analysis of adenoviral versus mRNA electroporation. Immature dendritic cells (DC) from monocytes and CD34⁺ cells were harvested on day 6 and transduced with AdV-GFP at a MOI of 1000 or electroporated with 30–50 μ g of mRNA-GFP. Cells were analyzed for GFP expression 24–48 hours after transfection. Flow cytometric analysis of GFP-transfected DC from (A) one patient and from (B) three individual experiments. Results are presented as mean fluorescence values (MFI) with standard deviation. MFI values for GFP negative population were subtracted. Monocyte derived DC transduced with AdV exhibit significantly higher GFP intensity as compared with AdV-transduced CD34⁺-derived DC ($p = 0.04$) or mRNA electroporated monocyte ($p = 0.02$).

compared with monocyte-derived DC, no notable difference in transgene expression was detected by either method.

Stimulatory Capacity of Transfected Dendritic Cells

Monocytes and CD34⁺ cells were differentiated into immature DC and transfected on day 6 with mRNA or by virus. Twenty-four hours later, these DC were added to allogeneic T cells for assessment of their stimulatory capacity. No decrease in DC stimulatory capacity was detected after transfection with mRNA electroporation (Fig. 5A). In some cases, at high stimulation ratio RV transduction suppressed the stimulatory capacity of

CD34⁺-derived DC, while monocyte- and to some extent CD34⁺-derived DC transduced with AdV exhibited enhanced stimulatory capacity (Fig. 5B). These results conclude that none of the different methods of gene delivery extensively inhibited the DC function.

We conclude that DNA is not suitable for transduction of DC using nonviral methods, while mRNA and square-wave electroporation yields reproducibly high levels of transduction in both subsets of DC, without disturbing the cell viability and retaining the stimulatory capacity. However, when comparing the amount of expressed protein per cell, AdV-mediated transfer is more efficient in monocyte-derived DC than mRNA electroporation.

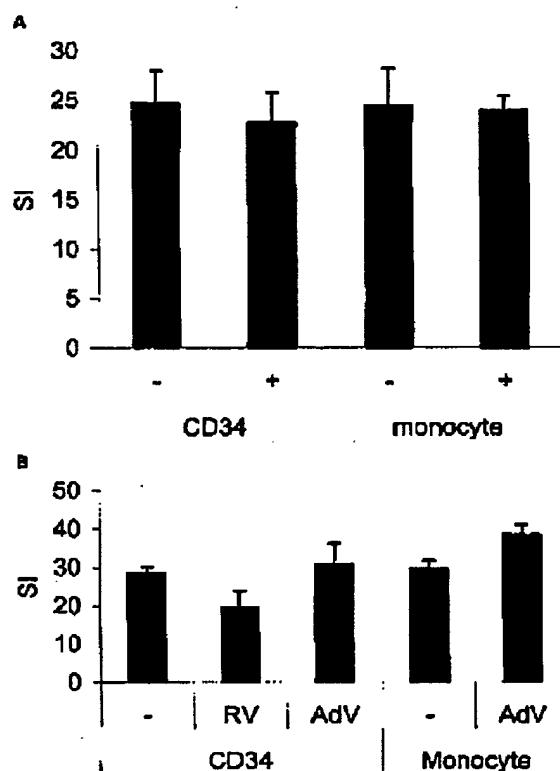


FIG. 5. Stimulatory capacity of monocyte- and CD34⁺-derived dendritic cells (DC). Monocyte- and CD34⁺-derived DC were differentiated into immature DC and transfected on day 6. Twenty-four hours after transfection, DC were cocultured with purified allogeneic T cells and uptake of ³H thymidine was measured after 5 days. Allogeneic T cells cocultured with (A) untransfected (–) or mRNA electroporated (+) at a responder:stimulator of 30:1. (B) Untransduced (–) or transduced with adeno (AdV) or retrovirus (RV) at a responder:stimulator of 75:1. Stimulation index (SI) is calculated by CPM values for the net stimulation divided by CPM values for responder cells alone. CPM values for stimulated cells ranged between 25000–100000. Results represent three different experiments. Error bars show standard deviation.

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DISCUSSION

We have compared clinically applicable strategies for transduction of human DC with regard to method of gene delivery, source of DC, and differentiation stage of cells. Contrary to the general view that high transgene expression in human DC may be achieved using viral vectors only, we demonstrate that a nonviral gene transfer method using square-wave electroporation and mRNA with a polyA tail yields similar efficacy of expression. This is the first study showing that nonviral transfection of DC derived from different source results in comparable numbers of transduced DC as when using virus-mediated transfer, with adenovirus (AdV)- and retrovirus (RV)-based vectors. The amount of expressed protein per cell is higher after AdV transduction than after mRNA electroporation. This difference is observed in monocyte-derived DC at high MOI only. Furthermore, by using cells from the same patient, we could detect great differences in the susceptibility to AdV-mediated gene transfer between CD34⁺ and monocyte-derived DC (22 ± 6% versus 65 ± 13%, respectively).

Nonviral methods are considered a safer alternative to virus-mediated gene transfer and have several important advantages for clinical application: (i) only the gene of interest is transcribed without immunologic interference from viral proteins; (ii) there is no risk of recombination associated with the viral vector; (iii) insertion of foreign DNA into the genome is not likely due to the transient nature of gene transfer; (iv) there is no need for cell proliferation, as with retroviral vectors; and (iv), in the case of DNA, it can be produced in large quantities and is very stable. These properties also make nucleic acids cheaper candidates for vaccines in clinical applications.

Controversy exists concerning DNA transfer into human DC (26–29). A recent study comparing the susceptibility with gene transfer by electroporation of naked DNA showed that monocytes differentiated into DC2, but not DC1 phenotype, were permissive to gene transfer (27). This might explain our negative results and those of others as well, because, under the generally used culture conditions (IL-4 and GM-CSF), preferentially type 1 DC are generated and characterized as being CD1a⁺.

However, square-wave electroporation with mRNA containing polyA tail and methylated 5'Cap resulted in up to 60% transfection efficiency. As compared with other studies that show that DC transfected with mRNA can stimulate CTL recognizing the encoded tumor antigens, despite the fact that the transgene expression was not detected at protein level (16,29,30), square-wave electroporation delivers sufficient amounts of mRNA to actually reveal protein expression for a minimum of 48

hours (22). Nevertheless, in this recent study, viral transduction might have resulted in superior transfection efficiency. Here we show by side-by-side comparison between nonviral and viral transfection methods of clinical grade DC from different sources that nonviral methods result in similar numbers of transfected cells as viral methods. Certain practical issues, such as, difficulties in isolation, capping, and in vitro transcription along with the inherent instability of RNA, would support the use of DNA over RNA. On the other hand, RNA has a safety advantage of not having the intrinsic capacity to integrate into the host genome.

It has been shown that DC transfected with RNA, either isolated from tumor tissue (16,31) or in vitro transcribed from a single specific gene (22,29,30,32–34), is capable of inducing a T-cell specific response. Either strategy has its advantages and limitations. We believe that although the use of a single gene is associated with the risk of inducing immunologic tumor-escape variants, at this stage of development of cancer vaccines, it is important to have an instrument for immunologic monitoring of the antitumor immune response and to have an unlimited source of the tumor antigen. The latter is often limiting in case of solid tumors, where large quantities of tumor tissue are required for RNA extraction. The batch variability of RNA preparations from different tumor specimens is a further "unknown," which makes the evaluation of immune response problematic. We are fully aware of, however, that the clinical future of cancer vaccines lies in the combination of multiple tumor antigens.

Regarding viral gene transfer, our results are consistent with previous reports demonstrating that: (i) human DC can be successfully transduced by RV or AdV vectors ex vivo, resulting in 40–90% transgene expression (20,35,36); (ii) DC are relatively refractory to adenoviral-mediated gene transfer at low MOI, but the transduction rate increases substantially at MOI of 100–1000 (37, 38); and (iii) RV vectors transduce proliferating cells, such as, CD34⁺-derived DC at low MOI (19,39,40).

One explanation for the requirement of high MOI for efficient AdV transduction is probably an inefficient viral attachment to the cell and internalization via the coxsackievirus and adenovirus receptor (CAR) and cell surface integrins, $\alpha v\beta 3$ and $\alpha v\beta 5$ (41–43). Studies have shown that monocyte-derived DC have low or absent expression of CAR as well as $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins (42,44). However, MHC class I molecules and $\alpha M\beta 2$ integrins can serve as alternative receptors for fiber-mediated and penton-mediated AdV attachment, respectively (44). The surprisingly large differences observed in the transfection efficiency of CD34⁺ versus mono-

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cyte-derived DC ($22 \pm 6\%$ and $65 \pm 13\%$, respectively) are probably a result of an insufficient expression of CAR on CD34⁺-derived DC (45).

Because the need for high MOI of AdV may restrict their administration *in vivo* (46), the alternative of transduced DC *ex vivo* may circumvent the limitations of administering free virus. Antiviral immunity should not be a hindrance, because it has been shown that repeated intramuscular administration of AdV is not limited by the presence of neutralizing antibodies in the serum (47). Moreover, Chirmule et al. (48) reported that half of patients, although having pre-existing immunity to AdV, did not generate immunity against the viral vector. On the contrary, anti-adenoviral immune response might act as an adjuvant to prime anti-transgene immunity, as has already been observed for adenoviral protein in the context of non-DC targeted vaccination strategies (49).

An advantage of RV over the use of AdV is not only the lower MOI and lack of antiviral immunity, but also their integration into the genome of the host. Therefore, RV-transduced DC may be able to constitutively express and process antigens to produce long-term antigen presentation. However, long-term expression that is a prerequisite for replacement gene therapy is not crucial for cancer vaccination strategies, where even a transient expression should be sufficient to prime an immune response.

Although a comparable number of transfected monocyte-derived DC is achieved by either method, AdV-transduced monocyte-derived DC display a significantly higher level of transgene expression. It was already shown that antigen-specific T cells can be generated by stimulation with RNA transfected DC, even if the transgene expression is not detected at the protein level (16, 26, 30). Therefore, it is not obvious that AdV-transduced DC are better stimulators of an antigen-specific T-cell response compared with mRNA electroporated ones, and this issue remains to be elucidated. Furthermore, previous reports have demonstrated that transduction, with certain viruses, leads to inhibition of stimulation capacity or even apoptosis of DC (50–52). We show that there is no significant downregulation of the stimulatory capacity by the transfected DC with either method.

In summary, the general belief is that the method of choice for transduction of DC is virally mediated gene delivery. In the present analysis comparing viral and nonviral methods to deliver genes into human DC, we show that mRNA transfection using square-wave electroporation results in a reproducibly comparable high percentage of transfected cells as compared with AdV- or RV-mediated gene delivery, without the inherent

safety concerns of viral vectors. This approach warrants for new exciting vaccination modalities in the clinic.

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